

FDA Contract 71-331, Evaluation of chemicals for toxic & teratogenic effects using the chick embryo as the test system  
No Date  
Butylated Hydroxy Toluene: FDA 71-25

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS  
USING THE CHICK EMBRYO AS THE TEST SYSTEM

BUTYLATED HYDROXY TOLUENE: FDA 71-25

WARF INSTITUTE, INC.  
MADISON, WISCONSIN

PS

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS  
USING THE CHICK EMBRYO AS THE TEST SYSTEM

BUTYLATED HYDROXY TOLUENE: FDA 71-25

WARF INSTITUTE, INC.  
MADISON, WISCONSIN

---

1

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS  
USING THE CHICK EMBRYO AS THE TEST SYSTEM

Objective: To determine the toxic and teratogenic effects of GRAS List compounds when injected into the air cell and yolk of fertile chicken eggs.

Procedure:

A. Test System and Incubation Procedures:

Fertile hatching eggs were chosen from a single comb white leghorn breeder flock. The eggs were candled and graded to eliminate internal and external defects; blood and meat spots, tremulous air cells, rough or cracked shells. The eggs chosen for injection weighed from 23 - 26 ounces/dozen and were not washed or dipped. The eggs were gathered within 48 hours of the injection or incubation and were held at 50 - 60°F. and 60 - 80% relative humidity. The breeder ration fed the flock was formulated by the breeder to meet or exceed the recommendations of the Nutrient Requirements of Poultry, Number 1 - 1971, National Academy of Sciences, and contained no additions of antibiotics, arsenicals, nitrofurazones or similar chemical additives. The breeder flock was blood tested and negative for pullorum-typhoid and mycoplasma gallisepticum.

Eggs were incubated in Jamesway 1080 forced air incubators equipped with automatic controls to regulate temperature, humidity and egg turning. Temperature and relative humidity were maintained at 99.5°F. and 86°F.\* wet bulb respectively for the first 18 days of incubation and eggs were turned each two hours. The eggs were then transferred to the hatcher in 3½" x 5" x 25" covered hardware cloth hatching baskets for the hatching period. Temperature in

\* 86°F. wet bulb refers to temperature of wet bulb apparatus in standard incubator hatching equipment and is equivalent to approximately 56% relative humidity.

the hatcher was maintained at 98.50F. and relative humidity at 860F. wet bulb. When the humidity had risen to 88 degrees as a result of moisture generated by hatching the hatcher was adjusted to hold 880F. wet bulb relative humidity until the chicks were removed on the morning of the 23rd day of incubation.

Prior to incubating or hatching each setting of eggs and following each hatching the incubator and its metal parts were thoroughly cleaned by vacuuming and washing with a 200 ppm solution of "ROCCAL" which contains 10% alkyl (C12, C14, C16 and related alkyl groups from C8 to C18) - dimethyl benzyl ammonium chloride. The sanitized surfaces were allowed to completely air dry prior to the introduction of eggs. Following each transfer of eggs to the hatching compartment, and when temperature and humidity had returned to normal levels, the hatcher and the eggs it contained were fumigated by combining 10 grams of potassium permanganate crystals and 20 grams of 37% formaldehyde solution.

#### B. Test Sample Preparation and Administration:

The test sample was taken up in an appropriate solvent to facilitate administration at the levels chosen. Sterile glassware, syringes and needles were employed to prepare and administer the test sample or solvent. Eggs previously selected were candled and the location of the air cell marked with pencil. The eggs were then randomized into the experimental groups. Injection of solvent or test sample dilution was accomplished by placing the material on the air cell membrane or by injection into the yolk sac. These administrations were made at both 0 and 96 hours of incubation. For the 0 hour experiments the eggs were assumed to be fertile; however, in the 96 hour experiments the eggs were candled as previously described and only those eggs with a well developed 96 hour embryo were selected for use.

##### 1. Air Cell Administration:

The eggs were wiped at the injection site with 70% ethanol and allowed to air dry. A hole measuring approximately 6mm was then drilled over the air cell in each egg using a "Dremel Moto-tool", model 270. The cutter employed deflected the shell fragments upwards and outwards. Remaining shell membrane fragments were removed with a small

forceps and the surface of the egg membrane visually examined for damage. The solvent or test sample was then deposited on the egg membrane with a model SB2 Syringe Microburet. Immediately following, the hole was sealed with  $\frac{1}{2}$ " Scotch Brand transparent tape. Two additional groups of eggs were normally included with each air cell experiment; a group which had been drilled, shell membrane fragments removed, and sealed only and a group of control eggs which had received no treatments whatsoever.

## 2. Yolk Administration:

The eggs were placed within a Fisher Scientific "Isolator/Lab" equipped with plastic irises through which the hands and forearms were placed during injection. Prior to injection the eggs and miscellaneous required equipment were submitted to a fumigation of 1.8 grams of potassium permanganate crystals and 3.6 grams of 37% formaldehyde. The eggs were held in this atmosphere for 30 minutes prior to further handling.

Each egg was then wiped at the injection site with 70% ethanol and allowed to air dry. A small hole was engraved directly over the air cell with a Burgess model V-13 Vibro-Graver. Care was taken not to damage the membrane attached to the shell. The surface of the egg at the engraved site was vacuumed to remove the shell particles produced. The egg was then slid onto the needle of the Syringe Microburet with the egg horizontal on its long axis until the top of the egg reached the hub of the  $\frac{1}{2}$ " - 25 ga. hypodermic needle. Following the injection of the material into the yolk sac, the egg was carefully withdrawn from the needle and the hole sealed with transparent tape. The hypodermic needle was carefully wiped with a sterile gauze pad prior to the next injection. As in the air cell administration, normally two additional groups of eggs were included in each yolk experiment; a group which had been drilled, pierced with the hypodermic needle and sealed only and a group of control eggs which had received no treatment other than fumigation.

Following the air cell and yolk injections the eggs were identified as to experiment and group with a No. 3 lead pencil and were then incubated as described above.

### C. Test Profile:

The work was divided into one or more Preliminary Range Finding Experiments, two Dose-Response and Teratogenic Experiments, and Ancillary Investigations (Post Hatch Trials).

#### 1. Preliminary Range Finding Experiments:

The objective of these trials was to locate the approximate LD-50 of the test sample. This data was used to design the dose levels for the Dose-Response trials. The test sample and solvent were administered by two routes; air cell and yolk, and at 0 and 96 hours of incubation. In general, at each route and time of incubation, 5 volumes of test sample dilution were administered together with 5 levels of solvent at the same volumes. Control eggs were also usually included as described above. Normally 10-20 eggs were used per group in these trials. When necessary these trials were repeated in an effort to locate the approximate LD-50 for the test compound.

Beginning on the 6th day of the incubation, the eggs set in the Preliminary Range-Finding Experiments were candled daily and non-viable embryos removed. These embryos were examined grossly for determination of developmental age and evidence of teratogenic effect, however, mortality was the main parameter in these trials. The remaining eggs were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The resultant chicks and non-viable embryos were examined grossly for teratogenic effects and all pertinent data was recorded. An estimate of the LD-50 for the test compound was then made.

#### 2. Dose-Response and Teratogenic Experiments:

Based upon information from the Preliminary Range-Finding Experiments, the Dose-Response Experiment was designed, employing 5 levels of sample dilution expected to produce mortality from the background level up through approximately 90%. Five volume levels of solvent were included as solvent controls at each route and time of administration. Normally 10 eggs/group were used in the solvent series with 50 eggs/group for the test sample dilutions. Twenty eggs/group were normally included for the drilled or pierced and non-treated controls. Two such experiments were conducted for each sample so that ultimately 100 eggs were tested on each test dilution at each route and stage of incubation.

5

The eggs set in these experiments were candled daily beginning on the 6th day of incubation and the non-viable embryos removed for examination as previously described. Where necessary, embryos were examined with the aid of a dissecting microscope. Remaining embryos were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The apparently normal chicks were then removed from the hatching trays and examined externally for anomalies.

Remaining non-viable embryos and chicks which were alive but unable to hatch were individually examined externally for abnormalities. These non-viable embryos, chicks which were alive but unable to hatch, and a portion of the normal chicks were examined in one aspect by X-ray. The chicks and embryos which had been X-rayed and all remaining normal chicks were then examined internally for possible anomalies of the viscera. All pertinent data were recorded.

### 3. Post Hatch Trials:

Apparently normal chicks were chosen from one 50 egg experiment for this portion of the study.

Generally 20 chicks (straight-run) were wing banded from each level chosen and were placed in Jamesway electrically heated battery brooders. Central Soya Chick Starter was fed as the sole ration to 8 weeks of age and Central Soya Grower from 8 weeks of age to termination. These diets were non-medicated. The chicks chosen were usually from the approximate LD-50 and no-effect levels for the test compound from each route of administration and time of incubation. Negative control, untreated chicks, were also included. In some cases chicks were chosen from groups where a relatively high incidence of anomalies were seen rather than from the LD-50 or no-effect levels specifically. Body weight data were collected weekly through 4 weeks of age and bi-weekly to termination. Average group feed consumption was recorded periodically.

### 4. Histopathology:

A random sampling of birds from selected groups were specified for histologic examination. These chicks comprised 5 males and 5 females from the test groups selected and 5 males and 5 females from a negative control group. Groups to be sampled were selected on the basis of observations of specific effects and a judgment made as to what groups would give the most information from the limited histopathologic examination.

The chicks sacrificed were either day old or varying ages in a Post Hatch Trial. The following tissues were collected, trimmed, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin:

1. Thyroid
2. Liver
3. Spleen
4. Pancreas
5. Lung
6. Heart
7. Kidney
8. Gonad
9. Bursa

The prepared slides were examined and remarkable alterations noted.

#### Results:

The data developed in the testing of Butylated Hydroxy Toluene are presented in the following tables:

#### Butylated Hydroxy Toluene

- Table 1 - Air Cell At 0 Hours
- Table 2 - Air Cell At 96 Hours
- Table 3 - Yolk At 0 Hours
- Table 4 - Yolk At 96 Hours
- Table 5 - Body Weight Data - Straight Run
- Table 6 - Histopathology

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administered milligrams per kilogram respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams).

Column 2 is the total number of eggs treated.

Column 3 is the percent mortality, i.e., total non-viable divided by total treated eggs.

Column 4 is the total number of abnormal birds expressed as a percentage of the total eggs treated. This includes all abnormalities observed and also toxic response such as

edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, ataxia or other nerve disorders.

Column 5 is the total number of birds having a structural abnormality of the head, viscera, limb, or body skeleton expressed as percentage of the total eggs treated. Toxic response and disorders such as those noted for column 4 are not included.

Column 2 through 5 have been corrected for accidental deaths if any occurred. Included in these columns are comparable data for the solvent-treated eggs and the untreated controls.

The mortality data in column 3 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose. The results are indicated at the bottom of each table.

The data of columns 3, 4 and 5 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels of percent mortality or percent abnormalities that are significant (probability of being the same is 5% or less) are indicated by an asterisk in the tables. All values so indicated have a higher incidence than the solvent values.

#### Discussion:

The comments and data which follow concern the results obtained when Butylated Hydroxy Toluene (BHT) was employed in the test system.

At 0 hour air cell, toxicity expressed as mortality was not significantly elevated in treatment groups receiving the test compound as compared with the solvent treated control. In 96 hour air cell treatment, the mortality responses were significantly elevated at dose levels of 88.0, 97.8 and 110.0 mg/kg. The LD-50 was 91.05 mg/kg. It was observed, however, that the combined solvent mortality for this time and route was 50.0%. Since the solvent mortality was elevated, its contribution to observed mortality in groups receiving BHT must be considered. As the mg/kg dose level increases, so also must the solvent volume increase as the various mg/kg dosages are administered from a single sample/solvent dilution.

In the 0 hour yolk treatments, there were significantly elevated levels of mortality seen. For the most part, these levels of mortality are not meaningful because of elevated mortality in the absolute ethanol control (24.1%) and in the pierced control (34.8%). As was

discussed previously, the treatment group mortality response must be evaluated including the solvent contribution to the total. The mortality slope was negative and the LD-50 was not calculable. At 96 hour yolk, mortality was significantly elevated only at the 293.3 mg/kg dose (55.0% mortality). The solvent control mortality for this time and route was 31.4%. The calculated LD-50 was 379.14 mg/kg.

Percent total abnormalities were significantly elevated in the 110.0 mg/kg, 96 hour air cell treatment. The primary condition seen was a temporary growth retardation which occurred during incubation. A single instance each of flexed mandible and crossed beak were also encountered, as well as 3 occurrences of parrot beak. These anomalies of the head had also been seen in the 3,315 egg flock background at low levels. (The flock background is total of all the drilled, pierced and untreated control eggs which were used in all the 50 egg studies using eggs from breeder flock N1). Several additional interesting anomalies were seen in the 500 eggs tested in 50 egg experiments in the 96 hour air cell treatments. Included were a single instance each of maxilla agenesis, buphthalmia, mouth dilation, ear atresia, leg phocomelia, leg micromelia, wing micromelia and foot ectrosyndact. These anomalies had not been observed in either the control eggs for this time or route nor had they been encountered in the flock background.

A significantly elevated level of total abnormalities was seen in a 10 egg range finding trial, 0 hour yolk 110.0 mg/kg. Two cases of retarded development were seen, one embryo with short mandible, and one embryo with short mandible and hair-like down. These anomalies had been encountered in the flock background. Also encountered among 0 hour yolk treatments were a single occurrence of long mandible, malformed head, anophthalmia and cornea agenesis. These anomalies were not seen in control eggs for this time and route nor had they been encountered in the flock background. Percent total abnormalities were not elevated in the 96 hour yolk treatments when compared to the solvent control.

A scattering of other abnormalities occurred at both 0 and 96 hours, air cell and yolk. Generally they were of a low level, had been seen in the control eggs for a particular time and route, and/or had been observed in the flock background.

Dwarfism, or retarded development, was a frequent observation in this experiment. To clarify our designation of dwarfism we will explain our classification procedure. At the first candling, day 5 or 6, we did not classify any embryos as retarded unless they were alive and definitely younger in development than 5 or 6 days. In subsequent candling any dead embryo judged by size to be 3 days behind in development was labeled as slight dwarfism, 4 days behind was labeled moderate dwarfism and 5 days or more behind was labeled severe dwarfism. If embryos were removed alive, 1 day behind was labeled slight, 2 days behind was labeled moderate and 3 days behind was labeled severe dwarfism. At hatch time an 18 day embryo was classified slightly dwarfed, a 17 day embryo was classified as moderate dwarfism and a 16 day embryo was classified as severe dwarfism.

One might suspect that at the toxic levels administered, embryo development could be delayed due to metabolic or nutritional alterations that produced temporary growth depression which would not result in a permanent growth defect. The retarded development was seen in the various solvent treated control eggs as well as the eggs receiving the test chemical. Additionally, the level of involvement in the solvent control eggs was only slightly less than the eggs receiving the test chemical. Chicks which hatched from BHT treated eggs were of normal size indicating that the growth depression seen during incubation was a toxic response to the solvent or test chemical rather than a teratogenic effect.

Post Hatch chicks were raised to 1 week of age and included birds from the untreated control and various treatment groups as indicated in the attached body weight table. Body weight gain and feed consumption were considered normal for all groups.

Five male and 5 female birds from the 96 hour air cell, 7.3 and 73.3 mg/kg, were sacrificed at approximately 10 days of age and examined grossly. No gross abnormalities were seen. Tissues from the chicks were subjected to histopathological examination and were compared with tissues of similar age, untreated controls. The tissue alterations seen were minimal in nature, generally low in incidence and fairly well distributed among the treatment and control groups.

X-ray examinations did not reveal abnormalities not already noted on gross observations.

#### Conclusions:

Under the conditions specified for this trial, Butylated Hydroxy Toluene was most toxic in 96 hour air cell administrations. The mortality at this time and route was dose related, however, the solvent contribution to observed mortality was considerable. In general, the abnormalities found in this experiment were of relatively low incidence and had been seen in control eggs for this experiment or in the flock background. In the 96 hour air cell and 0 hour yolk treatments however, anomalies of the head and limbs were seen which had not been found in control eggs for these times and routes or in the flock background. These anomalies were seen at very low levels, however, because of their unusual and severe nature, may suggest that further evaluation of the test sample is warranted.

Signed *R. B. Borden*

By and For WARF Institute, Inc.

November 14, 1974

Test Sample: Butylated Hydroxy Toluene

Identification: FDA 71-25

Solvent System: Absolute Ethanol

Breeder Flock: N-1

Preliminary Range Finding Experiments

<u>Experiment No.</u>	<u>Initiated</u>
30	5/30/72
35	7/03/72

Dose Response Experiments

<u>Experiment No.</u>	<u>Initiated</u>
38	7/24/72
48	9/25/72

Table 1

Butylated Hydroxy Toluene  
Air Cell At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
293.3	100	18.00	4.00	1.00
183.3	10	20.00	20.00	10.00
146.7	100	17.00	6.00	2.00
110.0	10	.00	.00	.00
110.0	10	10.00	.00	.00
97.8	10	10.00	.00	.00
88.0	9	11.11	11.11	.00
73.3	100	21.00	9.00	1.00
55.0	10	10.00	.00	.00
36.7	100	15.00	5.00	2.00
18.3	109	21.10	11.00	1.83
7.3	10	.00	10.00	.00
Absolute Ethanol	167	17.36	8.38	1.79
Drilled Control	60	11.66	3.33	.00
Control/ Control	190	9.47	2.63	1.05

\*\* Slope is negative, LD-50 not achieved

Table 2  
Butylated Hydroxy Toluene  
Air Cell At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
110.0	10	90.00*	20.00	20.00
110.0	110	81.81*	22.72*	5.45
97.8	10	90.00*	10.00	.00
88.0	10	90.00*	30.00	.00
73.3	110	58.18	17.27	4.54
36.7	110	38.18	17.27	8.18
18.3	110	11.81	5.45	3.63
7.3	110	10.90	2.72	.00
Absolute Ethanol	170	50.00	12.35	6.47
Drilled Control	50	8.00*	6.00	2.00
Control/ Control	190	9.47	2.63	1.05

\*\* LD-50 91.05 mg/kg

\* Significantly different from solvent ( $P \leq .05$ )

Table 3

Butylated Hydroxy Toluene  
Yolk At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
293.3	100	33.00	5.00	3.00
220.0	10	40.00	.00	.00
146.7	99	29.29	7.07	3.03
110.0	10	90.00*	40.00*	20.00
110.0	10	60.00*	.00	.00
97.8	10	60.00*	.00	.00
88.0	10	30.00	.00	.00
73.3	99	21.21	8.08	2.02
55.0	10	80.00*	.00	.00
36.7	110	28.18	9.09	.90
18.3	110	37.27*	10.90	1.81
Absolute Ethanol	170	24.11	5.88	3.52
Pierced Control	69	34.78	11.59	2.89
Control/ Control	190	9.47	2.63	1.05

\*\* Slope is negative, LD-50 not achieved

\* Significantly different from solvent ( $P \leq .05$ )

Table 4

Butylated Hydroxy Toluene  
Yolk At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
293.3	100	55.00*	9.00	2.00
220.0	10	40.00	20.00	20.00
146.7	100	34.00	17.00	2.00
110.0	10	20.00	.00	.00
110.0	10	10.00	.00	.00
97.8	10	30.00	10.00	10.00
88.0	10	40.00	10.00	10.00
73.3	100	25.00	20.00	3.00
55.0	10	20.00	.00	.00
36.7	98	35.71	16.32	6.12
18.3	109	19.26	7.33	.91
7.3	10	.00	10.00	.00
Absolute Ehtanol	169	31.36	21.30	3.55
Pierced Control	68	20.58	10.29	.00
Control/ Control	190	9.47	2.63	1.05

\*\* LD-50 379.14 mg/kg

\* Significantly different from solvent ( $P \leq .05$ )

Table 5

Body Weight Data - Post Hatch Response  
FDA 71-25: Butylated Hydroxy Toluene  
(Straight-Run)

<u>Test Dose (1)</u>	<u>Time/Route</u>	<u>Average Individual Body Weight - Grams Week 1</u>
-	Control/Control	82
293.3	O/AC	83
18.3	O/AC	87
293.3	O/Y	85
18.3	O/Y	82
73.3	96/AC	84
7.3	96/AC	76
146.7	96/Y	78
18.3	96/Y	80

(1) Milligrams/Kilogram Of Body Weight

Table 6  
Butylated Hydroxy Toluene  
Histopathology - Grow-Out Birds

<u>Histologic Observation</u>	<u>Negative Control (10)</u>	<u>96/AC 73.3 MG/KG (10)</u>	<u>96/AC 7.3 MG/KG (10)</u>
<u>Thyroid</u> cellular infiltration/increased cellularity less colloid present	2	8	2 2
<u>Lungs</u> congested	3		
<u>Heart</u> cellular infiltration	1		
<u>Spleen</u> pigmentation degeneration	8	9 2	10 2
<u>Liver</u> pigmentation cellular infiltration degeneration	7 9	2	1
<u>Small Intestine</u> lymphocytic infiltration		1	
<u>Colon</u> degeneration		1	
<u>Pancreas</u> degeneration	1	1	
<u>Kidney</u> degeneration atrophic glomeruli		1 1	1
<u>Testicles</u> immature cells degeneration	1 1	1 1	1 1